

# Direct regulation of *HSP60* expression by c-MYC induces transformation

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**Abstract** The *c-MYC* proto-oncogene encodes a ubiquitous transcription factor involved in cell proliferation and tumorigenesis. Heat shock protein 60 (HSP60) plays an essential role in assisting many newly synthesized proteins to reach their native forms. Increased HSP60 expression is observed in different types of human cancer. Here we show that c-MYC directly activates *HSP60* transcription through an E-box (CACGTG) site located in the proximal promoter of the *HSP60* gene. Overexpression of HSP60 induces transformation. Short-interference RNA (siRNA) mediated repression of HSP60 reduces transformation caused by c-MYC overexpression. These results indicate that c-MYC may promote transformation through the induction of *HSP60* expression.

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**Keywords:** c-MYC; HSP60; Transformation

## 1. Introduction

*c-MYC* proto-oncogene is involved in the control of cell cycle progression, proliferation, metabolism, and apoptosis [1,2]. The expression of c-MYC protein is tightly controlled during cell proliferation and differentiation [1,2]. *c-MYC* is one of the most frequently altered genes in human cancer and deregulation of *c-MYC* is constantly implicated in tumorigenesis [1,3,4]. Many c-MYC target genes were identified which are linked to proliferation, transformation, and metabolism [2]. Although many c-MYC target genes were identified, genes responsible for inducing transformation still remain to be explored.

Heat shock protein 60 (HSP60) is the major component of the chaperonins complex which weighs nearly a million Daltons and is composed of back-to-back rings of identical or closely related rotationally symmetric subunits [5,6]. HSP60 plays an essential role in assisting a large variety of newly synthesized proteins to reach their native forms by binding and facilitating their folding into native status inside a large central channel within each ring, as demonstrated in the bacterial GroEL–GroES–ATP complex [5–7]. However, folding of native proteins by a single domain was demonstrated in the mammalian HSP60 protein [8], which is different

from the bacterial model [5–7]. HSP60 was mostly shown to be involved in innate immunity and cardiac diseases [9,10]. Increased HSP60 expression is observed in different types of human cancers (ovarian cancer, pancreatic cancer, large bowel carcinoma, etc.) [11–13]. However, the role of HSP60 in tumorigenesis remains relatively unknown.

In this report, we demonstrated the tight correlation between c-MYC and HSP60 expression and direct regulation of *HSP60* expression by c-MYC through an E-box site located in the proximal promoter of the *HSP60* gene. Overexpression of HSP60 induces transformation in a Rat1a cell line. Short-interference RNA (siRNA) mediated repression of endogenous HSP60 expression in RatMyc cells causes a significant decrease in transformation. These results indicate that activation of *HSP60* expression by c-MYC is critical for c-MYC mediated transformation.

## 2. Materials and methods

### 2.1. Cell lines, plasmids, and reagents

The lymphoblastoid cell lines (CB33 control, CBMyc.Max, and CBMax), U937, EREB.TCMyc, Rat.MycER<sup>TM</sup>, RatMyc, 293T.Myc-si, and HeLaMyc-si cell lines were previously described [14,15]. The construction of different plasmids including the vector backbones, restriction sites, inserted fragments, and the oligonucleotides used in the construction of plasmids were described (Supplementary Tables 1 and 2). The generation of stable clones by calcium phosphate transfection was described [14]. All the stable clones were established by transfection of plasmids as designated by the name of the clones. The construction of pSUPER-siRNA plasmids were performed using the oligonucleotides as described (Supplementary Table 3). All the siRNA stable clones were designated by the suffix “-si”. 4-Hydroxytamoxifen and cycloheximide were previously described [14].

### 2.2. RNA purification, Northern blot analysis, protein extraction, and Western blot analysis

RNA purification and Northern blot analysis were previously described [14–16]. The HSP60 probe was generated from the full length cDNA of HSP60 (Supplementary Table 1). The  $\beta$ -actin, GAPDH, vimentin, c-MYC exon 1 and 3 probes were described [14–16]. The extraction of proteins from cells and Western blot analysis were performed as described [14,15]. The characteristics of the antibodies used were listed (Supplementary Table 4). Data shown here are representative of two or more experiments from independent cell cultures.

### 2.3. Cloning of the human *HSP60* promoter region, generation of *HSP60* promoter-driven reporter constructs, transient transfection, and luciferase assays

The *HSP60* promoter region (–541 to +16 bp surrounding the transcription start site) was cloned by PCR amplification of 293T genomic DNA and it was inserted into the *Hind*III/*Bgl*II sites of the pXP2 vector to generate the pHSP60PWLuc parental construct (Fig. 2B) [14,16]. The pHSP60PWLuc mutant construct was made by site-directed mutagenesis of the pHSP60PWLuc vector (Fig. 2B). The reporter

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**Abbreviations:** HSP60, heat shock protein 60; ChIP, chromatin immunoprecipitation

constructs were co-transfected into 293T cells with pMT2T, pMT2TMyC, or its deletion mutants [14–16]. A pCMV- $\beta$ -gal plasmid was used as an internal control. The luciferase activities were assayed as described [14].

#### 2.4. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed as described [15]. Briefly, cells were cross-linked and harvested. The lysates were incubated with no antibody or antibodies specific for c-Rel or c-MYC. The DNA fragments were extracted and resuspended for PCR analysis. The PCR reaction generated a 220 bp product from *HSP60* proximal promoter (–550 to –331 bp) containing E-box (–523 to –518 bp), two 220 bp products from the *HSP60* intron 1 region containing E-boxes (+232 to +237 bp, +460 to +465 bp), or a 265 bp product from the distal promoter region (–1844 to –1580 bp) without E-box. The regions in the *HSP90A* gene promoter which were used in the control ChIP experiments were described [17]. The primers and antibodies used in the ChIP assays were listed (Supplementary Tables 3 and 4).

#### 2.5. Soft agar colony formation assay

The stable clones were plated at three different cell density ( $5 \times 10^3$ ,  $10^4$ ,  $2 \times 10^4$ ) using standard assay conditions as mentioned except that 15% fetal calf serum was used [15,17]. Data shown here are representative of two or more experiments from independent cell cultures.

### 3. Results

#### 3.1. Correlation between c-MYC and HSP60 expression

To identify c-MYC target genes involved in transformation, we screened a cDNA microarray filter (Atlas Human array, Clontech) using cDNA probes generated from a previously

characterized B cell line (EREb.TCMyc) [14–17]. Among numerous genes overexpressed in c-MYC expressing cells, we focused on the gene *HSP60* due to the increased HSP60 expression in different tumor types [11–13]. Northern blot analysis showed that withdrawal of estrogen and tetracycline (causing induction of exogenous c-MYC) caused a 2-fold increase in the expression of *HSP60* (Fig. 1A). *HSP60* expression significantly increased (~2.5-fold) in CBMyc.Max cells when compared to the control lymphoblastoid cells (CB33 control) or CBMax cells (Fig. 1B). Western blot analysis showed that the increase in HSP60 protein expression also coincided with the increase in its mRNA levels (Fig. 1C). To confirm this correlation in different cell types, mRNA levels of *HSP60* were shown to decrease in U937 cells induced into differentiation by TPA, which correlated with the decline in c-MYC expression (Fig. 1D). Rat HSP60 protein levels also increased (~2-fold) in RatMyc cells overexpressing c-MYC compared with the control RatCMV cells (Fig. 1E). To test whether *HSP60* expression is regulated by endogenous c-MYC, we performed siRNA experiments on 293T and HeLa cells to knockdown endogenous c-MYC expression [15,17]. The results showed that endogenous c-MYC expression was repressed in the 293TMyC-si and HeLaMyc-si clones followed by a corresponding decrease in the HSP60 protein levels compared to the control 293T and HeLa clones (Fig. 1F and G), demonstrating the regulation of *HSP60* by endogenous c-MYC. All the results demonstrated the tight correlation between c-MYC and *HSP60* expression.

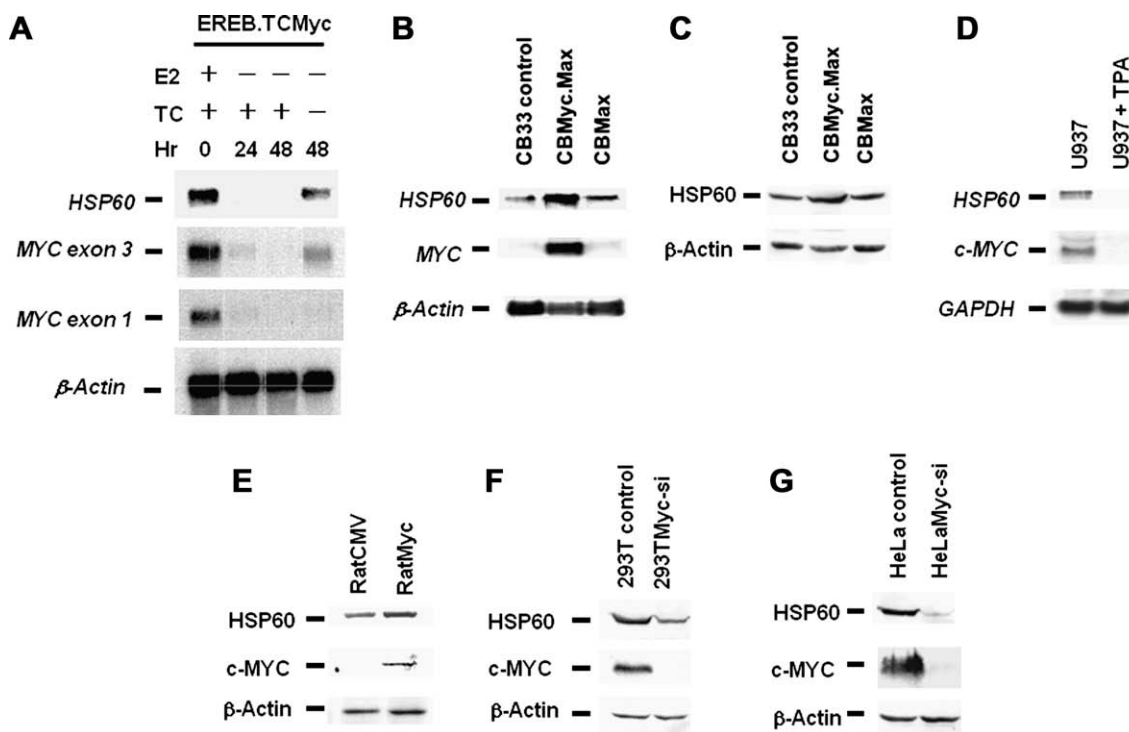


Fig. 1. Activation of *HSP60* expression by c-MYC in a variety of cell lines. (A) Northern blot analysis of RNA from EREb.TCMyc cells upon estrogen (E2) withdrawal (lanes 2–4) and c-MYC activation by tetracycline (TC) withdrawal (lane 4) showed the activation of *HSP60* expression by c-MYC. The same blot was sequentially hybridized with the indicated probes (see Section 2). (B) Northern blot analysis of RNA from different CB33 lymphoblastoid cell lines (CB33 control, CBMyc.Max, and CBMax) showed the correlation between c-MYC and *HSP60* gene expression. The same blot was sequentially hybridized with the indicated probes. (C) Western blot analysis of the cell lysates from the CB33 control, CBMyc.Max, and CBMax cells using an anti-HSP60 antibody and an anti- $\beta$ -actin antibody as a control for protein loading. (D) Correlation of *HSP60* vs. c-MYC expression in U937 cells with or without TPA induction by Northern blot analysis. (E) Increased rat HSP60 protein levels in Rat1a cells overexpressing c-MYC (RatMyc) vs. the control RatCMV cells by Western blot analysis. (F) and (G) Western blot analysis showed the decrease in HSP60 levels in the 293T or HeLa clone expressing siRNA against c-MYC (293TMyC-si or HeLaMyc-si) vs. the control 293T or HeLa clone.

### 3.2. Direct regulation of *HSP60* expression by c-MYC

To investigate whether c-MYC-mediated upregulation of *HSP60* expression was direct, we studied *HSP60* expression in a Rat.MycER<sup>TM</sup> cell line previously described [14,15,17]. By treating the cells with 4-hydroxytamoxifen (TM), which specifically activates MycER<sup>TM</sup>, and by simultaneous treatment with cyclohexamide (CX) to inhibit new protein synthesis, Fig. 2A showed that TM treatment led to a significant (~4-fold after 4 h) and rapid (detectable after 1/2 h) increase in *HSP60* mRNA levels (left panel). This induction cannot be abolished by co-treatment with CX (CX + TM) (Fig. 2A, middle panel), indicating that induction of *HSP60* expression involves a mechanism that is independent of de novo protein synthesis in the presence of MycER proteins and is consistent with a direct effect of c-MYC. To ascertain whether c-MYC binds to the *HSP60* promoter, we investigated whether the *HSP60* promoter contains c-MYC binding sites. An E-box site (CACGTG) was identified ~1.5 kb upstream from the *HSP60* translation start site (ATG) (Fig. 2B). A reporter vector con-

taining the *HSP60* promoter with the E-box site (pHSP60-PWLuc; Fig. 2B) was then co-transfected with either the control pMT2T or the pMT2TMyC expression vector into 293T cells. Fig. 2C showed that c-MYC was able to activate the expression of pHSP60PWLuc about 2.5-fold. However, c-MYC was not able to activate the pHSP60PMLuc construct whose E-box was mutated to CACCTG (Fig. 2B and C), indicating that the E-box in the *HSP60* promoter responded to transcriptional activation by c-MYC. In addition, the c-MYC mutants lacking the transactivation domain [pMT2TMyC(Δ7–91)] or heterodimerization domain [pMT2TMyC(Δ371–412)] were defective in activating the expression of pHSP60PWLuc (Fig. 2D). However, the Myc BoxII deletion mutant [pMT2TMyC(Δ122–140)] was able to activate the expression of pHSP60PWLuc (Fig. 2D). These results indicated that c-MYC-mediated activation of *HSP60* transcription required both the transactivation and heterodimerization domains, but not the Myc BoxII domain [18]. To determine whether c-MYC binds to the E-box in vivo, a ChIP assay using

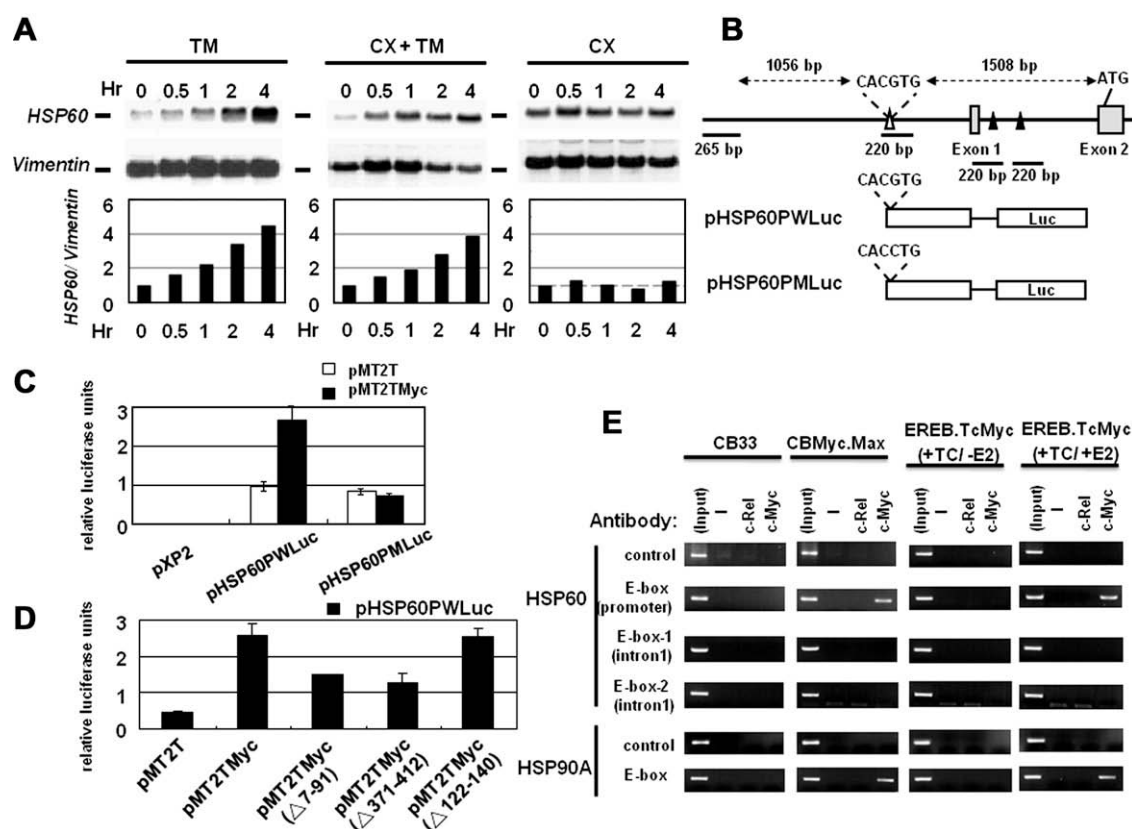


Fig. 2. Direct regulation of *HSP60* expression by c-MYC. (A) Northern blot analysis of Rat.MycER<sup>TM</sup> cells upon activation of MycER<sup>TM</sup> by 4-hydroxytamoxifen (TM; 500 nM) alone, by TM in the presence of cyclohexamide (CX; 10 μg/ml), or with CX alone. The same blots were sequentially hybridized to a *HSP60* probe and a *vimentin* probe as a control for RNA loading. Quantification of the ratio of *HSP60* vs. *vimentin* was shown underneath each panel. (B) Schematic representation of the genomic region encompassing the 5' flanking region of the human *HSP60* promoter and the reporter constructs used for analysis of transcriptional activation by c-MYC. E-boxes located in the proximal promoter and intron 1 (represented by open and closed triangles) were depicted. The reporter constructs (pHSP60PWLuc and pHSP60PMLuc) were made as described (Section 2). (C) Transcriptional activation of pHSP60PWLuc, but not pHSP60PMLuc, by c-MYC using transient transfection assays. (D) Co-transfection of pHSP60PWLuc and expression vectors of c-MYC wild type or its different deletion mutants showed that the transactivation or heterodimerization deletion mutant was defective in activating pHSP60PWLuc using transient transfection assays. (E) PCR amplification of immunoprecipitated chromatin prepared from different cell lines. Chromatin was incubated without antibody (–), with an anti-c-Rel (c-Rel) or with an anti-c-MYC (c-MYC) antibody. Different columns represented experiments using different cell lines such as CB33 control cells, CBMyc.Max cells, EREB.TCMyc(+TC/–E2) cells, and EREB.TCMyc(+TC/+E2) cells. The lengths of different PCR fragments were shown in Supplementary Table 3. The labeling of control represented the promoter fragments in the *HSP60* or *HSP90A* gene without E-box. Different E-boxes represented the E-box located in the promoter region of *HSP60* or *HSP90A* gene or in the intron 1 region of *HSP60* gene. Input is 2% of total input lysate.

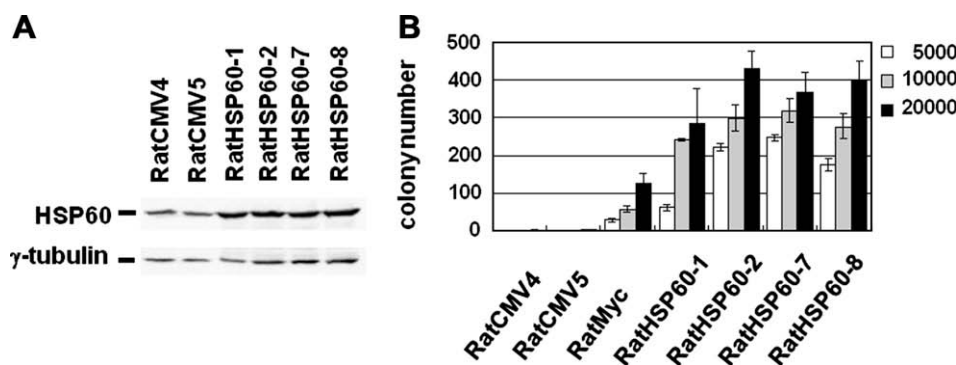


Fig. 3. Overexpression of HSP60 induces transformation in a Rat1a cell line in vitro. (A) Western blot analysis showed the increased HSP60 protein levels in Rat1a clones overexpressing HSP60 (RatHSP60 clones). (B) RatHSP60 clones had increased soft agar colony formation activity compared to Rat1a control clones (RatCMV). The numbers (5000, 10000, 20000) in the inset indicated the number of cells plated in each plate. Error bars indicated standard deviations (S.D.).

CBMyc.Max cells was performed (Fig. 2E under the CBMyc-Max column). The results showed that c-MYC, but not c-Rel, bound to the E-box as demonstrated by the c-MYC immunoprecipitated chromatin amplifiable to generate a 220 bp fragment (depicted in Fig. 2B and labeled as E-box (promoter) in Fig. 2E). Control experiment to amplify another *HSP60* promoter fragment (265 bp as depicted in Fig. 2B) which did not contain an E-box failed to generate an amplifiable fragment (labeled as control in Fig. 2E). The 220 bp fragment was also amplifiable from the c-MYC immunoprecipitated chromatin prepared from EREB.TCMyc(+TC/+E2) cells, demonstrating the binding of endogenous c-MYC to the E-box site (Fig. 2E under the EREB.TCMyc(+TC/+E2) column). In addition, the 220 bp fragment was not amplifiable in c-MYC immunoprecipitated chromatin prepared from CB33 control cells or quiescent EREB.TCMyc(+TC/-E2) cells

(Fig. 2E under the CB33 or the EREB.TCMyc(+TC/-E2) column). A positive control experiment using the E-box located in the *HSP90A* gene promoter [17] showed a similar intensity of amplified PCR fragment by ChIP experiment (labeled as E-box in Fig. 2E under the HSP90A section). Taken together, these results indicated that c-MYC directly activates *HSP60* transcription by binding to the E-box site located in the *HSP60* proximal promoter.

### 3.3. Induction of transformation by HSP60 in a Rat1a cell line

To test whether HSP60 contributes to transformation activity caused by c-MYC, Rat1a stable clones overexpressing HSP60 were generated (Fig. 3A). Soft agar colony formation assay was performed. The results showed that Rat1a clones overexpressing HSP60 (RatHSP60) had increased soft colony formation activity compared to the control Rat1a clones (Rat-

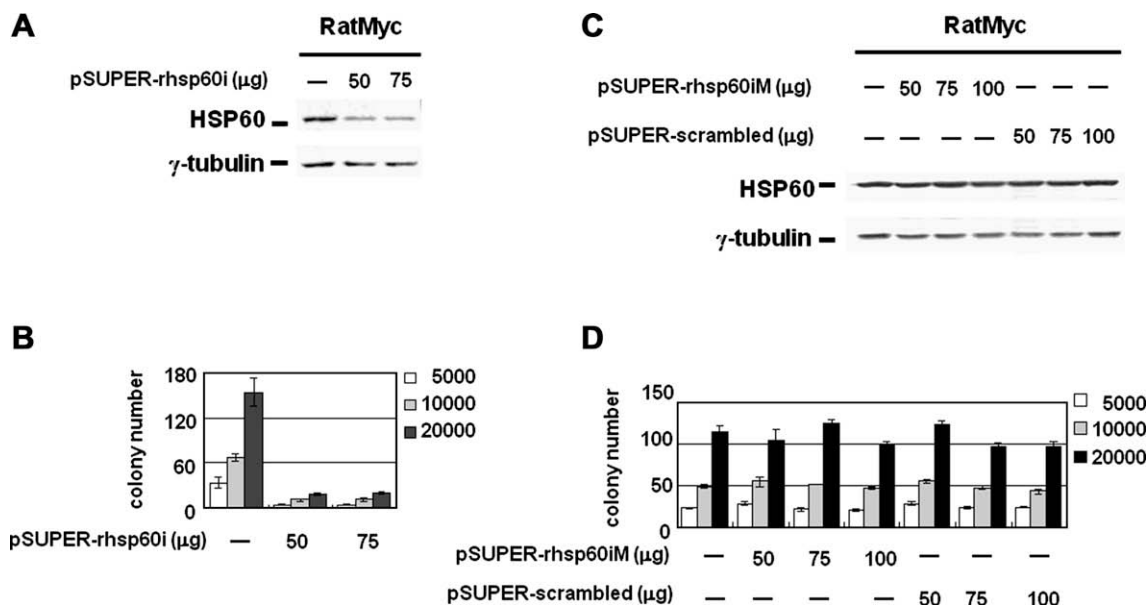


Fig. 4. The critical role of HSP60 in the transformation activity of RatMyc cells. (A) Western blot analysis showed the decreased HSP60 levels in RatMyc cells receiving siRNA against endogenous HSP60. Different amounts of pSUPER-rhsp60i plasmids were transfected into RatMyc cells. (B) Significantly decreased soft agar colony formation activity in RatMyc cells receiving siRNA against endogenous HSP60. The numbers (5000, 10000, 20000) in the inset indicated the number of cells plated in each plate. Error bars indicated standard deviations (S.D.). (C) Western blot analysis showed no significant decrease in HSP60 protein levels in RatMyc cells receiving mismatched or scrambled siRNA as controls. (D) No significant decrease in soft agar colony formation activity in RatMyc cells receiving mismatched or scrambled siRNA as controls. The numbers (5000, 10000, 20000) in the inset indicated the number of cells plated in each plate. Error bars indicated standard deviations (S.D.).



CMV) (Fig. 3B), supporting the role of HSP60 in the induction of transformation mediated by c-MYC.

### 3.4. The critical role of HSP60 in c-MYC mediated transformation

To determine whether the role of HSP60 is critical in the induction of transformation caused by c-MYC, RatMyc cells were transiently transfected with pSUPER-rhsp60i plasmid to repress endogenous HSP60 (Fig. 4A). Soft agar colony formation assays were performed comparing the RatMyc cells receiving siRNA against HSP60 vs. the control RatMyc clone. The results showed that there was a significant decrease in transformation activity in RatMyc cells receiving siRNA against HSP60 (Fig. 4B), demonstrating the critical role of HSP60 in the induction of transformation by c-MYC. Control experiments using pSUPER plasmids expressing mismatched siRNA (pSUPER-rhsp60iM) or scrambled siRNA (pSUPER-scrambled) did not cause a decrease in HSP60 protein levels (Fig. 4C) or transformation activity of RatMyc cells (Fig. 4D), supporting the specificity of pSUPER-rhsp60i plasmid used to repress endogenous HSP60 in RatMyc cells. These results demonstrated the critical role of HSP60 in c-MYC mediated transformation.

## 4. Discussion

Collectively, our results demonstrated that *HSP60* expression is regulated by c-MYC and overexpression of HSP60 induces transformation. *HSP60* was shown to be a putative c-MYC target gene [19–23]. Our detailed characterization using different cell lines and identification of an E-box site in the proximal promoter of the *HSP60* genes further demonstrated that *HSP60* is a direct c-MYC target gene. Although the Myc BoxII deletion was shown to impair most of the transactivation activity mediated by wild type Myc, it was shown that a small proportion of c-Myc target genes could still be activated by Myc BoxII deletion mutant [18]. From our results, it was possible that *HSP60* may belong to this kind of target genes not requiring the transactivation function of Myc BoxII domain (Fig. 2D). Soft agar colony formation assays supported its possible role in c-MYC mediated transformation. siRNA mediated repression of HSP60 in RatMyc cells significantly decreased the transformation activity. *HSP60* activation may be a crucial component of the program by which constitutive c-MYC expression induces transformation.

Increased HSP60 expression is observed in different types of human cancers (ovarian cancer, pancreatic cancer, large bowel carcinoma, etc.) [11–13]. However, the role of increased HSP60 expression in tumorigenesis is relatively unknown. Our demonstration that overexpression of HSP60 induces soft agar colony formation in a Rat1a cell line is consistent with its role to contribute to transformation and tumorigenesis in different types of human cancers. Recently, HSP60 was shown to stabilize mitochondrial survivin and inhibit apoptosis in tumor cells, which also supports the tumorigenic role of HSP60 [24]. HSP60 was mostly shown to be involved in innate immunity and cardiac diseases [9,10] in contrast to our demonstration of its role in transformation. Taken together, our results demonstrated the direct regulation of *HSP60* expression by c-MYC and the critical role of HSP60 in c-MYC mediated transformation.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.febslet.2008.11.004](https://doi.org/10.1016/j.febslet.2008.11.004).

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